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TITLE: Novel mTORC1 and 2 Signaling Pathways in Polycystic Kidney Disease (PKD)

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13. SUPPLEMENTARY NOTES

Abstract: This proposal will study novel mTORC1 and 2 signaling pathways that mediate ADPKD and investigate the effects of mTORC1 (Raptor) knockout, mTORC2 (Rictor) knockout or combined mTORC1 and 2 knockout on cyst growth and kidney function. The overall hypothesis is that there is increased mTORC1 (4E-BP1) and mTORC2 (AktSer473, PKCα and SGK1) signaling in PKD kidneys and that combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice will slow cyst growth and improve kidney function more than mTORC1 (Raptor) knockout or mTORC2 (Rictor) knockout alone. We have made significant progress in the first year: We have characterized 4E-BP1 signaling pathways in PKD kidneys and cells. We have developed Pkd1-/-, mTORC2 (Rictor) -/- double knockout mice that demonstrate less cysts than Pkd1 -/- mice alone. We have 24 mice in an ongoing experiment to compare the therapeutic effect of the mTOR kinase inhibitor Torin-2 (that inhibits both mTORC1 and mTORC2) with sirolimus (that inhibits mTORC1) on cyst growth and kidney function. We have used FISP-MRI scanning to obtain measurements of kidney and cyst volume in live PKD mice.

15. SUBJECT TERMS

Polycystic kidney disease, PKD, mTORC1, mTORC2, Raptor, Rictor.

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1. **INTRODUCTION:**

ADPKD is the most common life threatening hereditary disease in the USA. ADPKD accounts for about 5-10% of end-stage renal failure in the USA requiring dialysis and renal transplantation. There is no effective treatment for ADPKD. This proposal will study novel mTORC1 and 2 signaling pathways that mediate ADPKD and investigate the effects of mTORC1 (Raptor) knockout, mTORC2 (Rictor) knockout or combined mTORC1 and 2 knockout on cyst growth and kidney function. The overall hypothesis is that there is increased mTORC1 (4E-BP1) and mTORC2 (AktSer473, PKCα and SGK1) signaling in PKD kidneys and that combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice will slow cyst growth and improve kidney function more than mTORC1 (Raptor) knockout or mTORC2 (Rictor) knockout alone. These studies will unequivocally address whether the mTORC1 (4E-BP1) or mTORC2 pathway or both pathways is important in PKD and whether it is worthwhile performing therapeutic interventions using the novel mTORC1 and 2 inhibitors that are in clinical trials in humans

2. **KEYWORDS:**

Autosomal dominant polycystic kidney disease

mTORC1

Raptor

mTORC2

Rictor

4E-BP1

mTOR kinase inhibitor

3. **ACCOMPLISHMENTS: What were the major goals of the project?**

Major Task 1: To determine the effect of mTORC1 (Raptor) knockout in Pkd1 -/- mice

Subtask 1: To determine whether Raptor knockout decreases the cystic phenotype
Subtask 2: To determine the effect of Raptor -/- on 4E-BP1 signaling
Subtask 3: To determine the effect of 4E-BP inhibition on PKD

Major Task 2: To determine the effect of mTORC2 (Rictor) knockout in Pkd1 -/- mice

Subtask 1: To determine whether Rictor knockout reduces the cystic phenotype in vivo

Subtask 2: AktSer473, SGK1 and PKCα phosphorylation in PKD in vivo

Subtask 3: AktSer473, SGK1 and PKCα phosphorylation in PKD in vitro

Major Task 3: To determine the effect of combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice

Subtask 1: To determine the effect of combined mTORC1 (Raptor) knockout and mTORC2 (Rictor) knockout in Pkd1 -/- mice on apoptosis and proliferation, cyst growth and kidney function.

SubTask 2: To determine the effect of TORKs versus sirolimus on PKD and kidney function

What was accomplished under these goals?

(1) Major activities:

Major Task 1: Development of Pkd1 -/- Raptor -/- double knockout mice. 4E-BP1 signaling in PKD studies.

Major Task 2: mTORC2 (Rictor) knockout in Pkd1 -/- mice.

Major task 3: Treatment of Pkd1 -/- mice with mTOR kinase inhibitors that inhibit mTORC1 and mTORC2 (TORKs) versus Sirolimus (mTORC1 inhibitor).

Major Tasks 1, 2 and 3: Use of FISP-MRI scans to determine polycystic kidney volume and cyst volume and number of cysts per kidney at different time points of the disease.

2) Specific objectives:

Major Task 1: To determine whether Raptor knockout decreases the cystic phenotype. To determine the effect of Raptor -/- on 4E-BP1 signaling

Major Task 2: To determine whether Rictor knockout reduces the cystic phenotype in vivo.

Major Task 3: To determine the effect of TORKs versus sirolimus on PKD and kidney function

3) Major results:

Major Task 1: We have obtained Raptor floxed mice and are in the process of breeding the Raptor floxed mice with mice with a kidney-specific tamoxifen-inducible Pkd1 Cre mice to develop Raptor-/-, Pkd1 -/- double knockout mice. We are in the first stage of the breeding process.

Major task 1: A major finding is that we have determined that phosphorylation insensitive 4E-BP1 reduces the hyperproliferative phenotype in vitro. The abstract below summarizes these findings:

Background: Unchecked proliferation of cystic epithelial cells is a major contributor to cyst growth in PKD. The 4E-BP1 pathway is a crucial checkpoint in protein translation initiation and cellular proliferation. Evidence

from oncology supports the malignant potential of 4E-BP1. A recognized oncotarget, 4E-BP1 is associated with worsening progression, metastasis, and morbidity in oncology. The aim of this study was to determine 1) whether PKD patient and animal model kidney tissues have dysregulated phospho 4E-BP1 species and 2) the effect of a phosphorylation insensitive 4E-BP1 (F113A) on phospho 4E-BP1 species distribution, cap dependent protein translation, and proliferation in renal epithelial cells.

Methods: Immunofluorescence staining of phospho 4E-BP1 species (T70, T37/47, S65) was performed on human ADPKD and Han:SPRD rat (Cy) kidneys. Western blot analysis, Cyquant cellular proliferation, and Firefly-renilla assays were performed on human primary epithelial cells from normal renal cortical tubular epithelium ($PKD1^{+/+}$) and ADPKD cyst-lining epithelium ($PKD1^{-/-}$) transfected with control or pCAG-F113A or transduced with control or F113A lentivectors.

Results: Phospho 4E-BP1 species were present in cyst lining cells of human ADPKD and Cy renal tissues. F113A resulted in substantially reduced phospho 4E-BP1 T37/46(0.89±0.08 vs 0.012±0.004DU, p<0.01) and S65 (0.63±0.04 vs 0.003±0.001DU, p<0.01), reduced cap-dependent protein translation (37%, p<0.01), and reduced 72hr proliferation (250±4 vs 180±5 480/528nm O.D, p<0.0001) in $PKD1^{-/-}$ cells. Surprisingly, in $PKD1^{+/+}$cells, F113A resulted in no phospho 4E-BP1 reduction, reduced cap-dependent protein translation (32%, p<0.01), and marginally reduced proliferation (375±5 vs 314±4 480/528nm O.D, p<0.0001). Acute stimulation with insulin resulted in maintained S65 suppression with F113A transfection in $PKD1^{-/-}$ (2.1±0.3 vs 0.2±0.1AU, *p<0.0001).

Conclusions: F113A overexpression results in a shift towards hypophosphorylated 4E-BP1 species in the presence or absence of stimulation, reduced cap dependent protein translation, and reduced proliferation, with more aggressive effects in $PKD1^{-/-}$ cells. Utilizing F113A gene therapy to counter the loss of the translationally repressive 4E-BP1 pathway in a murine model of PKD, is the next step in addressing a pathway seemingly integral to the pathobiology of PKD.

<u>Major Task 2: To determine whether Rictor knockout reduces the cystic phenotype in vivo</u>

Another major finding is that we have determined that mTORC2 (Rictor) knockout decreases the cystic phenotype in Pkd1 -/- mice. The abstract below summarizes these findings.

Background: mTOR exists in two distinct structural and functional complexes mTORC1 (Raptor) and mTORC2 (Rictor -Rapamycin-independent companion of mTOR). We have shown that mTOR kinase inhibition that inhibits both Raptor and Rictor) reduces cyst growth. However, the effect of mTORC2 (Rictor) inhibition alone on PKD is not known. The aim of the study was to determine the effect on the cystic phenotype of mTORC2 (Rictor) knockout in Pkd1 -/- mice

Methods: Expression of kidney specific Cre recombinase, with Tamoxifen on days 19-21, in Pkd1 fl/fl mice results in Pkd1 -/- mice that have a slow onset of cystic disease with severe PKD and renal failure at 130 days after tamoxifen injection . To determine if absence of Rictor reverses the cystic phenotype of Pkd1 -/- mice, Rictor fl/fl mice were bred with the Pkd1 fl/fl; KspCad-CreERT2 mice to develop Rictor fl/fl, Pkd1 fl/fl; KspCad-CreERT2 mice that were treated with tamoxifen to develop kidney-specific Rictor, Pkd1 double -/- mice that were also evaluated at 130 days post tamoxifen. No of cysts per kidney at 90 days, a time when cysts are just beginning to form, was counted in live mice by FISP-MRI scan

Results: (See Table 1)

Table 1	Wild type	Pkd1 -/-	Pkd1 -/- Rictor -/-
Body wt (g)	30	27	32
2K (g)	0.35	0.53	0.36 *
2K/TBW (%)	1.1	2.0	1.2 *
Cyst volume (%)	0	40.1	14.6 *
No of cysts/kidney	0	5.3	0.8 *
Heart wt (g)	0.3	0.15	0.17
BUN (mg/dL)	24	29	25
SCr (mg/dL)	0.22	0.33	0.2 *

2K/TBW (%) Two kidney/total body weight, *P<0.05 vs. Pkd1 -/-

Summary: mTORC2 (Rictor) -/- in Pkd1 -/- mice results in significantly lower kidney weight, cyst volume, number of cysts and SCr

Conclusions: Study of signaling pathways downstream of mTORC2 (pAkt, SGK1, PKCα) and a head to head study of sirolimus (Raptor inhibitor) VS. new generation mTOR kinase inhibitors (inhibit both Raptor and Rictor) will be interesting.

<u>Major Task 3: To determine the effect of TORKs versus sirolimus on PKD and kidney function.</u>

We have started treating a group of 21 Pkd 1-/- mice with either vehicle, sirolimus (mTORC1 inhibitor) or Torin-2 (mTORC1 and mTORC2 inhibitor). We will perform MRI scans of kidneys every 2 months to

determine kidney and cyst volume. At 150 days of age, mice will be sacrificed for determination of kidney function, cyst volume and mTORC1 and 2 signaling pathways

4) Other achievements: Major tasks 1, 2 and 3.

We have used FISP-MRI scanning to obtain measurements of kidney and cyst volume in live PKD mice at 70 days of age (See Figure 1). FISP-MRI will allow us to perform longitudinal imaging of cysts and determination of kidney volume over a period of time in the same animal (Figure 1).

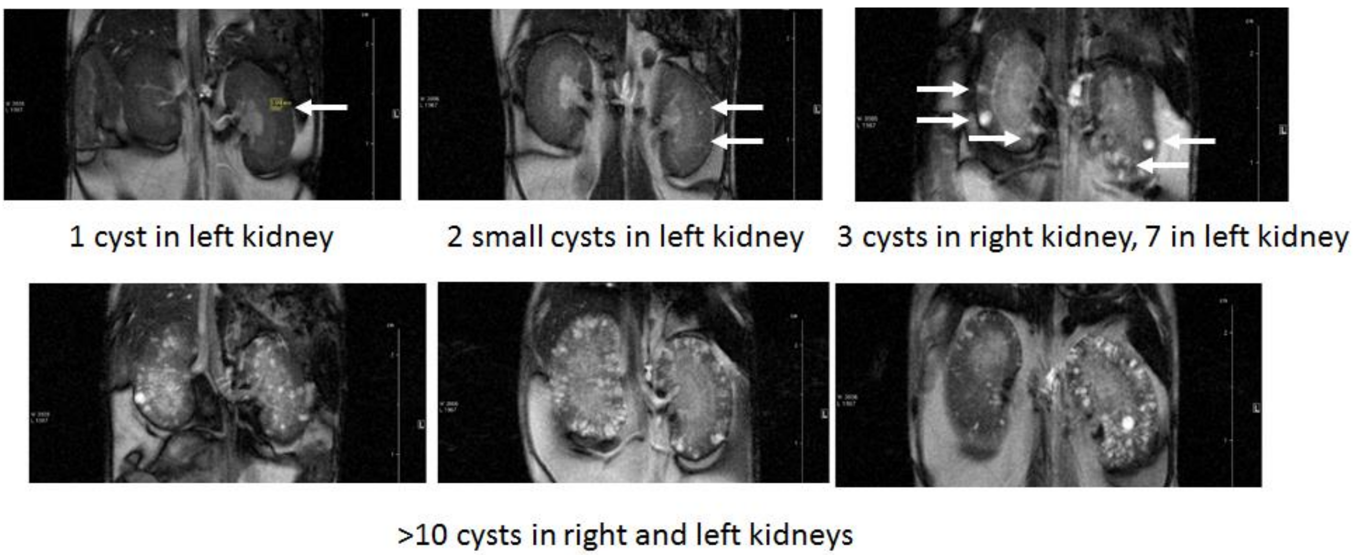

Fig 1: Fast imaging with steady-state precision-magnetic resonance imaging (FISP-MRI) provides a rapid, sensitive and accurate method to quantitatively measure cyst, organ and parenchymal volumes in Pkd1 -/- mouse kidneys.

What opportunities for training and professional development has the project provided?

The grant was not meant to provide training or professional development. However, during the summer there were two high school students and 2 college students working in the Laboratory as volunteers to advance their professional skills in laboratory medical research. Dr Holditch attended the FASEB PKD Conference in Montana in June 2017. Dr Holditch presented a poster at this conference, got feedback on the poster and attended lectures that resulted in increased knowledge and skill in the area of PKD.

How were the results disseminated to communities of interest?

Our results to date will be presented at the annual American Society of Nephrology Meeting in New Orleans in Novermber, 2017.

What do you plan to do during the next reporting period to accomplish the goals?

1) We have obtained Raptor floxed mice and are in the process of breeding the Raptor floxed mice with mice with a kidney-specific tamoxifen-inducible Pkd1 Cre mice to develop Raptor-/-, Pkd1 -/- double knockout mice. We are in the first stage of the breeding process.

2) We are performing pilot studies to use a novel intervention; utilizing adeno-associated viral vectors expressing the F113A 4E-BP1, in a murine model of PKD. F113A 4E-BP1 is a mutant that cannot be phosphorylated and should block the 4E-BP1 signaling pathway in PKD kidneys.

3) We will increase the numbers of Pkd1 -/- Rictor -/- double knockout mice and perform MRI scans every 2 months in these mice and at sacrifice measure kidney function, cysts volume and mTORC1 and mTORC2 signaling pathways. We shall confirm that Rictor is knocked out in the double knockout mice and measure mTORC2 signaling pathways (AktSer473, PKCα, SGK1) in the double knockout mice

4) We will treat a group of 21 Pkd 1-/- mice with either vehicle, sirolimus (mTORC1 inhibitor) or Torin-2 (mTORC1 and mTORC2 inhibitor) and determine the effects of therapy on PKD and kidney function and store kidneys for study of mTORC1 and 2 signaling pathways..

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

A major finding is that we have determined that phosphorylation insensitive 4E-BP1 reduces hyperproliferative phenotype in vitro. Use of this phosphorylation-insensitive 4E-BP1 may provide a future rationale for treatment to decrease PKD

Another major finding is that we have determined that mTORC2 (Rictor) knockout decreases the cystic phenotype in Pkd1 -/- mice. These findings provide a rationale for the use of combined mTORC1 and 2 inhibitors (the mTOR kinase inhibitors or TORKs) as future treatments in PKD

What was the impact on other disciplines?

Many cancers are driven by increased 4E-BP1 signaling. Use of this phosphorylation-insensitive 4E-BP1 may provide a future rationale for treatment to decrease certain cancers.

Many cancers are driven by increased mTORC2 signaling. Demonstration that mTORC2 inhibition decreases proliferation and has an effect on apoptosis may provide a future rationale for the development of specific mTORC2 inhibitors treatment to decrease certain cancers.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Unfortunately, there is no effective FDA-approved treatment for ADPKD.

The genetic studies (Pkd1 -/- mice with additional knockout of mTORC1 or 2 or both) will offer mechanistic insights into novel mTORC1 and 2 signaling pathways and the failure of rapalogues in PKD.

4E-BP1 is a proliferative signaling molecule downstream of mTORC1 that is not inhibited by rapalogues. AktSer473, PKCα and SGK1 are signaling molecules downstream of mTORC2. The mTOR kinase inhibitors (TORKs) inhibit both mTORC1 and 2 and are in human studies in cancer. The pharmacological studies (4E-BP1 inhibition, AktSer473, PKCα or SGK1 inhibition and TORKs) will provide insights into future therapies for PKD.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

No significant changes were made.

Actual or anticipated problems or delays and actions or plans to resolve them

The first Professional Research Assistant (PRA) hired on the project did not work out. There was a delay of 2 months in between which the first PRA resigned and a new PRA was hired. The lack of efficiency of the first PRA and the training of the new PRA led to some delay in the project. There were no other delays in the project

Changes that had a significant impact on expenditures

Delays in hiring PRA staff and periods when there was no PRA.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None.

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals.

None

Significant changes in use of biohazards and/or select agents

None

PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations

Nothing to report (yet)

Journal publications.

Nothing to report (yet)

Books or other non-periodical, one-time publications.

Nothing to report (yet)

Other publications, conference papers, and presentations.

Two abstracts accepted for presentation at the annual American Society of Nephrology meeting in New Orleans in November, 2017

1) mTORC2 (Rictor) knockout decreases the cystic phenotype in Pkd1-/- mice

2) Phosphorylation insensitive 4E-BP1 reduces hyperproliferative phenotype in vitro

Website(s) or other Internet site(s)

Nothing to report (yet)

Technologies or techniques

Nothing to report (yet)

Inventions, patent applications, and/or licenses

Nothing to report (yet)

Other Products

Nothing to report (yet)

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Example:

Name:	*Charles Edelstein*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	Dr. Edelstein has overseen the design, performance and analysis of the studies
Funding Support:	DOD funding
Name:	*Sara Holditch*
Project Role:	*Post-doctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*4E-BP1 studies, Creation of double knockout mice, Mouse treatment studies,*
Funding Support:	DOD funding

Name:	Michelle Vasquez
Project Role:	PRA
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Genotyping animals, Performing immunoblots.
Funding Support:	
Name:	*Dan Attwood*
Project Role:	*PRA*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Genotyping animals, Immunoblots, animal injections*
Funding Support:	
Name:	*Nicole Brown*
Project Role:	*PRA*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Genotyping animals, animal injections, Performing immunoblots, General Lab Manager duties*
Funding Support:	VA Merit Award

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

VA Merit Award VA Merit Award to Charles Edelstein . 1I01BX001737-01A1. The IL-33/ST2/CD4 cell system in acute kidney injury. Period: 01/01/13-01/01/17. No Cost extension 01/01/2017-01/01/2018

What other organizations were involved as partners?

Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Not applicable

QUAD CHARTS:

Not applicable

APPENDICES:

Attached are abstracts that have been accepted as poster presentations for the annual American Society of Nephrology meeting in New Orleans in November 2017.

mTORC2 (Rictor) knockout decreases the cystic phenotype in Pkd1$^{-/-}$ mice

Holditch S, Brown N, Ravichandran K, Edelstein CL

Background: mTOR exists in two distinct structural and functional complexes mTORC1 (Raptor) and mTORC2 (Rictor-Rapamycin-independent companion of mTOR). We have shown that mTOR kinase inhibition, capable of inhibiting both Raptor and Rictor, reduces cyst growth. However, the effect of mTORC2 (Rictor) inhibition alone on PKD is not known. The aim of the study was to determine the effect of Rictor$^{-/-}$ on the cystic phenotype of Pkd1$^{-/-}$ mice

Methods: Expression of kidney specific Cre recombinase, with Tamoxifen administration on days 19-21, in Pkd1 fl/fl mice results in Pkd1$^{-/-}$ mice that have a slow onset of cystic disease with severe PKD and renal failure at 130 days post tamoxifen injection. To determine if the effect of Rictor$^{-/-}$ on the cystic phenotype of Pkd1$^{-/-}$mice, Rictor fl/fl mice were bred with the Pkd1 fl/fl; KspCad-CreERT2 mice to develop Rictor fl/fl, Pkd1 fl/fl; KspCad-CreERT2 mice treated with tamoxifen to develop kidney-specific Rictor$^{-/-}$Pkd1$^{-/-}$ mice and aged to 150 days post Tamoxifen. Non-invasive quantitative assessment of cyst development per kidney at 90 days, a time when nascent cysts are forming, was performed by T2-weighted and FISP-MRI at 4.7 Tesla.

Results: Genetic deletion of mTORC2 (Rictor) in Pkd1$^{-/-}$mice results in significantly lower kidney weight, cyst volume, number of cysts and SCr. (See Table)

	Wild type (n=8)	Pkd1$^{-/-}$ (n=9)	Pkd1$^{-/-}$ Rictor$^{-/-}$ (n=6)
Body wt (g)	30	27	32
2K (g)	0.35	0.53	0.36*
2K/TBW (%)	1.1	2.0	1.2*
Cyst volume (%)	0	40.1	14.6*
No of cysts/kidney	0	5.3	0.8*
Heart wt (g)	0.3	0.15	0.17

BUN (mg/dL)	24	29	25
SCr (mg/dL)	0.22	0.33	0.2*

2K/TBW (%) Two kidney/total body weight, *p<0.05 vs. Pkd1$^{-/-}$

Conclusions: Study of signaling pathways downstream of mTORC2 (pAkt, SGK1, PKCα) and a head to head study of sirolimus (Raptor inhibitor) VS. new generation mTOR kinase inhibitors (inhibit both Raptor and Rictor) is warranted to better understand the pathways responsible for PKD cyst expansion.

Phosphorylation insensitive 4E-BP1 reduces hyperproliferative phenotype in vitro

Sara J Holditch[1], Carolyn N Brown[1], Kameswaran Ravichandran[2], Charles L Edelstein[1]
[1]*Univ Colorado Denver;* [2]*Hera BioLabs*

Background: Unchecked proliferation of cystic epithelial cells is a major contributor to cyst growth in PKD. The 4E-BP1 pathway is a crucial checkpoint in protein translation initiation and cellular proliferation. Evidence from oncology supports the malignant potential of 4E-BP1. A recognized oncotarget, 4E-BP1 is associated with worsening progression, metastasis, and morbidity in oncology. The aim of this study was to determine 1) whether PKD patient and animal model kidney tissues have dysregulated phospho 4E-BP1 species and 2) the effect of a phosphorylation insensitive 4E-BP1 (F113A) on phospho 4E-BP1 species distribution, cap dependent protein translation, and proliferation in renal epithelial cells.

Methods: Immunofluorescence staining of phospho 4E-BP1 species (T70, T37/47, S65) was performed on human ADPKD and Han:SPRD rat (Cy) kidneys. Western blot analysis, Cyquant cellular proliferation, and Firefly-renilla assays were performed on human primary epithelial cells from normal renal cortical tubular epithelium (PKD1$^{+/+}$) and ADPKD cyst-lining epithelium (PKD1$^{-/-}$) transfected with control or pCAG-F113A or transduced with control or F113A lentivectors.

Results: Phospho 4E-BP1 species were present in cyst lining cells of human ADPKD and Cy renal tissues. F113A resulted in substantially reduced phospho 4E-BP1 T37/46(0.89±0.08 vs 0.012±0.004DU, p<0.01) and S65 (0.63±0.04 vs 0.003±0.001DU, p<0.01), reduced cap-dependent protein translation (37%, p<0.01), and reduced 72hr proliferation (250±4 vs 180±5 480/528nm O.D, p<0.0001) in PKD1$^{-/-}$ cells. Surprisingly, in PKD1$^{+/+}$ cells, F113A resulted in no phospho 4E-BP1 reduction, reduced cap-dependent protein translation (32%, p<0.01), and marginally reduced proliferation (375±5 vs 314±4 480/528nm O.D, p<0.0001). Acute stimulation with insulin resulted in maintained S65 suppression with F113A transfection in PKD1$^{-/-}$ (2.1±0.3 vs 0.2±0.1AU, *p<0.0001).

Conclusions: F113A overexpression results in a shift towards hypophosphorylated 4E-BP1 species, reduced cap dependent protein translation, and reduced proliferation, with more aggressive effects in PKD1$^{-/-}$ vs PKD1$^{+/+}$ cells. Utilizing F113A gene therapy to counter the loss of the translationally repressive 4E-BP1 pathway in a murine model of PKD, is the next step in addressing a pathway seemingly integral to the pathobiology of PKD.